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Mitchell et al., Mutat. Res. 70:91-105, 1980 Vol. 79, #2

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Irene Marx
Art Unit 1651
CMI 10-E-05,
Mail Box 11-B-01
703-308-2922

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MUTAGENICITY OF ANTIBIOTICS IN MICROBIAL ASSAYS

PROBLEMS OF EVALUATION

I. deG. MITCHELL, P.A. DIXON, P.J. GILBERT and D.J. WHITE

Beecham Pharmaceuticals Research Division, Medicinal Research Centre, The Pinnacles, Harlow, Essex (Great Britain)

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Summary

5 antibiotics, 4 of which inhibit protein synthesis in different ways, and 1 of which inhibits bacterial cell-wall synthesis, were tested in a battery of microbial assays for possible genetic effects.

All the antibiotics, chloramphenicol, tetracycline, gentamicin, oleandomycin and phosphonomycin induced forward mutation to L-azetidine-2-carboxylic acid resistance in *Escherichia coli* WP2. This response was closely correlated with the toxic effects and was inferred to be deletion mutation. In addition, chloramphenicol was weakly active in reversion of the frame-shift mutation in *Salmonella typhimurium* TA98, gentamicin caused petite induction in *S. cerevisiae* at pH 4.4—4.7 and tetracycline gave a significant response with gene conversion and petite induction also in *S. cerevisiae* but at pH 7.2.

The results, particularly those with *E. coli*, cast doubts on the validity of testing specifically designed antibacterial agents in bacteria, and raise serious problems in the evaluation of such data in terms of risk to human populations.

The testing of antibacterial agents presents theoretical and practical problems for mutagenicity assays in a bacterial screen. Indeed, the validity of using

Abbreviations: 9AA, 9-amino-acridine; A2C, L-azetidine-2-carboxylic acid; ACR, acriflavine; ade, adenine; 2AF, 2-amino-fluorene; amp, ampicillin; BP, benzo[*a*]pyrene; bio, biotin; CAP, chloramphenicol; CP, cyclophosphamide; DMCB (DMCA), Davis—Mingioli slats broth (agar) for *E. coli*; DMSB, Davis—Mingioli salts broth for *Salmonella*; *DMSO*, dimethylsulphoxide; DR, daunorubicin; EOH, ethanol; GM, gentamicin; HIA, heart infusion agar; his, histidine; MMS, methyl methanesulphonate; MC, mitomycin C; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; NA, nutreint agar; NB2, nutrient broth number 2; OM, oleandomycin; PM, phosphonomycin; TC, tetracycline hydrochloride; trp, tryptophan; VBA, Vogel—Bonner agar; YCB (YCA), yeast complete broth (agar); YNBA, yeast nitrogen-base agar.

bacteria at all in assays on agents which are specifically toxic (either inhibitory or lethal) to bacteria is questionable.

Protagonists of bacterial assays might argue that such assays are entirely valid unless there is a species-specific effect on the DNA of the tester organism. However, cellular metabolism consists of a series of interlinked biochemical cycles so that a toxic effect on one process might eventually affect DNA metabolism in a non-specific, quantitative manner. Thus, it could be argued that any observed genetic effect might have resulted indirectly from the toxic effects. In the case of antibiotics this toxicity would be peculiar to bacteria and any bacterial mutagenicity would therefore be irrelevant to mammals.

Of equal importance to the theoretical aspects is the practical problem of testing antibacterial chemicals with bacteria. The Ames agar-plate assay has already proved unsatisfactory for several antibiotics [10]. Furthermore, it is unlikely that the fluctuation test [7] would detect agents where the mutagenicity was consequent upon a high degree of toxicity. However, the development of a sensitive modification of the "treat and plate" method [12] has enabled antibacterial agents to be adequately tested at moderate concentrations in this study.

We theorized that if antibacterial agents produced quantitative, indirect effects on DNA, these would be most likely to be lethal, or to take the form of deletion mutation. This form of mutation cannot be detected by normal reversion assays [1,6] so we have included forward mutation to resistance to L-azetidine-2-carboxylic acid, $A2C^r$, (previously described in some of the literature as c'-azetidine carboxylic acid resistance, $caca^r$) [11,13] and a repair test [15] (both carried out using *E. coli*) in addition to reversion assays. Also gene conversion [21] and petite induction in yeast [14,15] have been assayed to test effects in a eucaryotic organism.

5 antibiotics have been assayed; 4 were different classes of inhibitors of protein synthesis (chloramphenicol, tetracycline, gentamicin and oleandomycin), whilst one was thought to be specific for inhibition of cell wall synthesis (phosphonomycin).

Materials and methods

Strains used

Salmonella typhimurium strains TA98 and TA100 were obtained from Prof. B.N. Ames, University of California (U.S.A.).

Escherichia coli strains WP2 and WP100 were obtained from Prof. B.A. Bridges, M.R.C., Cell Mutation Unit, University of Sussex, Brighton (Great Britain).

Saccharomyces cerevisiae strain D4 was obtained from Dr. B.J. Kilbey, Genetics Department, University of Edinburgh (Scotland).

Mutagens, antibiotics and selective agents used

BP, 2AF, MNNG, ACR, 9AA, MC, A2C and CAP were obtained from Sigma. TC and OM came from Pfizer, GM from Nicholas Labs. and PM from the Spanish Penicillin and Antibiotic Co. CP, MMS and DR were obtained from Wellcome Labs., Eastman and Calbiochem resp.

All agents were dissolved in sterile, distilled water except for high concentrations of CAP (>100 μg/ml) which were dissolved in DMSO, and 9AA which was dissolved in EOH.

Basic media used

Bacterial minimal broths were prepared as by Mitchell [12]. Minimal agars (DMCA and VBA) were prepared as by Bridges [2] and Vogel and Bonner [19]. For *E. coli* media the glucose content was 0.25% and for Salmonella 2.0% (w/v). Minimal agar for yeast (YNBA) was obtained from Difco.

Complete agars and broths for bacteria were obtained from Oxoid (NB2 and NA) or Difco (HIA). Yeast complete agar (YCA) and broth (YCB) were made as by Mitchell [11] normally with an adenine addition (30.0 μg/ml). Standard YCA was weakly buffered to approx. pH 5.8 using 0.02% (w/v) Na_2HPO_4 and 0.015% citric acid. pH 7.2 YCA was made by substituting this buffer with 0.7% Na_2HPO_4 and 0.2% KH_2PO_4, whilst pH 4.4–4.7 YCA was buffered with 0.5% Na_2HPO_4 and 0.5% citric acid.

Genetic systems and assay methods used

(a) *The repair test* was performed with repair-efficient and repair-deficient strains of *E. coli*, resp. *uvrA*$^+$ *recA*$^+$ (WP2) and *uvrA*$^-$ *recA*$^-$ (WP100).

Overnight cultures of the strains were diluted to give 10^4 cells/ml (WP2) or 10^5 cells/ml (WP100) and 0.5 ml of these respective suspensions were added to one or other of a pair of 100-ml conical flasks. Additionally each conical flask contained 2.75 ml NB2, 0.60 ml saline (buffered to pH 7.4), 0.90 ml 0.15 M KCl or S9 mix (for microsomal activation) and 0.25 ml of experimental or control agent.

Cultures were shaken reciprocally (110 cycles/min, 1.0 cm amplitude) for 3.0 h at 37°C, then diluted 10^{-1}, 10^{-2} and 10^{-3}; 0.10 ml of each dilution were spotted onto duplicate NA petri dishes and colonies were counted after 24-h incubation at 37°C.

In this assay concentration of test agent was arranged to give between 2- and 5-fold inhibition of strain WP2.

(b) *Tryptophan reversion and forward mutation to A2C*^{r} were assessed in *E. coli* WP2.

About 4 colonies from a stock culture petri-dish were placed in each of 5 9.0-ml aliquots NB2 which were incubated for 4.0 h at 37°C. Each culture was then added to a 100-ml conical flask containing 20.0 ml NB2. These cultures were incubated, shaken, at 37°C for 17.0 h after which they were centrifuged (2000 *g* for 15 min) and the pellets combined and resuspended in saline to give 1.50×10^{10} cells/ml. 1.0-ml aliquots were added to 100-ml conical flasks which contained 2.85 ml modified DMCB [12] supplemented with 20.0 μg/ml tryptophan, 0.90 ml 0.15 M KCl or S9 mix (for microsomal activation) and 0.25 ml of experimental or control agent.

Cultures were incubated, shaken, for 1.50 h at 37°C then centrifuged and the pellets resuspended in 15.0 ml NB2. There was a further 2.0 h incubation in conical flasks, shaken, at 37°C after which the cultures were recentrifuged, the pellets washed once in 9.0 ml saline and resuspended in 1.0 ml saline. This suspension was then diluted to give 2.0×10^{10} cells/ml.

TABLE 1

ACCUMULATED DATA FOR THE SPONTANEOUS FREQUENCIES OF MUTATION FOR THE ASSAYS USED

Species	Strain	System	Total tests	Spontaneous frequency data					Average increases over the control required for significance at	
				Denom.	Av. freq.	Median freq.	Interval est. ($p \triangleq 0.05$) on median	Variance coefficient (within sets of tests) (%)	$p \triangleq 0.05$	$p < 0.001$
s. typhimurium	TA98	*his*$^-$ → *his*$^+$	53	10^9	26.4	13.3	11.2—16.0	17.8	1.4	1.9
E. coli	WP2	*trp*$^-$ → *trp*$^+$	10	10^9	10.5	4.5	2.3—32.7	27.1	1.7	2.3
		A2C^S → A2C^r	13	10^7	36.4	24.8	15.1—60.0	15.4	1.4	1.8
	WP2 and WP100	*uvrA*$^+$ *recA*$^+$ cf. *uvrA*$^-$ *recA*$^-$	10	—	—	—	—	21.8	1.5	2.1
S. cerevisiae	D4	*trp*$^-$ → *trp*$^+$	25	10^6	18.0	15.2	12.2—28.4	13.3	1.3	1.7
		p$^+$ → *p*$^-$	25	10^3	3.0	1.9	1.3— 3.3	70.2	1.9	3.7

TABLE 2

QUANTITIES OF KNOWN MUTAGENS REQUIRED TO GIVE A SIGNIFICANT RESPONSE IN THE REPAIR TEST *E. coli* AND IN PETITE INDUCTION *S. cerevisiae*

Species	Assay system	Agent tested and concentration (μg/ml)									
		2AP	MMS	MNNG	HP	MC	DR	9AA	[a] CP	[a] ACR	[a] 2AF
E. coli	*uvrA*$^+$ *recA*$^+$ cf. *uvrA*$^-$ *recA*$^-$	NR	NT	<20.0	NR	<0.4	<0.5	NR	[b] 500—1000	<5.0	20.0
S. cerevisiae	*p*$^+$ → *p*$^-$	NR	0.58 μl/ml	1.15	12.0	100—200	60.0	NR	NT	<25.0	NT

[a] Requiring microsomal activation.
[b] Inconsistent result.
NR, no response.
NT, no test.

Tryptophan reversion was assayed by spreading 4.0×10^9 cells/plate on each of 3 DMCA plates and forward mutation ($A2C^r$) by spreading 2.0×10^7 cells/plate on each of 3 DMCA plates supplemented with 20.0 μg/ml trp and 50.0 μg/ml A2C. Colony-forming units (survival) were determined by plating 10^2 cells on each of 3 NA petri dishes. Colonies were counted after about 43 h incubation.

Highly toxic concentrations of agent could be assayed by this method (Tables 4—13).

(c) *Histidine reversion* assays were carried out with *S. typhimurium* TA98 in essentially the same way as tryptophan reversion in *E. coli*. The main differences were that the inocula were prepared in NB2 containing 40.0 μg/ml ampicillin, modified DMSB was used in place of DMCB and histidine reversion was determined by plating 2.0×10^9 cells on each of 3 VBA petri dishes supplemented with 20.0 μg/ml biotin.

(d) *Gene conversion (trp) and petite induction* were assayed with *S. cerevisiae* D4 at low (4.4—4.7) and high (7.2) pH.

A suspension was made from stock cultures and spread onto YCA petri dishes at 10^2 cells/plate. After 72 h incubation at 28°C about 16 colonies were suspended in 9.0 ml of saline and 3.0-ml aliquots were added to each of two 100-ml flasks containing 30.0 ml YCB. These cultures were incubated, shaken, at 28°C for 17 h after which they were centrifuged and resuspended in saline to give 4.0×10^7 cells/ml. 1.0 ml was then added to each 100-ml conical flask which also contained 8.5 ml YCB and 0.5 ml of experimental or control agent.

After 5 h incubation, shaken, at 28°C cultures were centrifuged, the pellets washed once in 9.0 ml saline and resuspended in 1.0 ml saline. This suspension was then diluted to give 2.0×10^7 cells/ml.

Gene conversion (*trp*) was assayed by spreading 2.0×10^6 cells on each of 3 YNBA petri dishes supplemented with 30.0 μg/ml adenine; petite induction (pale brown usually small colonies) [14,16], and survival were assayed by spreading 2×10^2 cells on each of 9 YCA petri dishes lacking the usual adenine supplement.

As the antibiotics under test were relatively non-toxic to yeast, up to 4500 μg/ml were assayed in these tests.

Growth in cultures during tests

Cell counts were performed in duplicate by haemacytometer; in general a 2—3-fold increase in numbers was observed during the incubation period in the negative control samples in the mutation assays used here. Growth in the repair test approached 10^2-fold.

Control tests

In every assay the control frequency (Fc) was the mean of duplicate negative controls (solvent) which were treated identically to the test samples. A positive control was also included in every assay.

Preparation and optimization of S9 microsomal mix

The S9 mix was prepared as by Ames et al. [1] (with Aroclor 1254 stimulation of the rats). The level of microsomes in the mix was optimized by tests with 3 standards: 10 μg/plate BP, 20 μg/plate 2AF and 2000 μg/plate CP in

TABLE 3

SUMMARY OF THE RESULTS

Agent tested	Responses in the strains and genetic systems used					
	E. coli WP2			*S. typhimurium* TA98	*S. cerevisiae* D4	
	trp$^-$ → *trp*$^+$ (Base subs.)	A2C^S → A2C^r (forward mutation)	*uvr*A$^+$ *rec*A$^+$ cf. *uvr*A$^-$ *rec*A$^-$ (repair)	*his*$^-$ → *his*$^+$ (frame-shift)	*trp*$^-$ → *trp*$^+$ (conversion)	*p*$^+$ → *p*$^-$ (induction)
Chloramphenicol	—	++ (10.0)	—	+ (9.0)	—	?
Tetracycline	—	++ (2.3)	—	—	++ (175.0) [a]	++ (≅1000) [a]
Gentamicin	—	+++ (0.49)	—	—	—	+++ (≅500) [b]
Oleandomycin	—	?+ (399.0)	—	?	—	—
Phosphonomycin	NT	+++ (3.3)	—	—	—	—

[a] high pH only.
[b] low pH only.
NT, no test.
—, no response.
?, doubtful response.
+, weak response ($p < 0.01$) but *Qm* or *RI* <5.0.
++, moderate response *Qm* or *RI* between 5.0 and 15.0
+++, strong response *Qm* or *RI* >15.0.
( ), concentration of agent required to give a result significant at $p < 0.01$ in μg/ml.

agar plate assays with *S. typhimurium* strain TA100 [1]. When converted to μl microsomes per ml of top agar, results from these tests are directly applicable to the liquid culture tests used here [12].

All bacterial assays were carried out with and without microsomal activation. However, activation was thought to be unnecessary with yeast which possesses its own oxidases [3].

Assessment of results of mutation and conversion assays

The increase in mutants which is required to give a significant response

TABLE 4

MUTATION TO A2C^r IN *E. coli* WITH CHLORAMPHENICOL

Conc. of CAP (μg/ml)	Expt. No.	Inhibition Total cfu con. / Total cfu Expt.	Percent survival cfu/plate Expt. X 100 / cfu/plate con.	*Fe* − *Fc* [a] per plate	*Fe* − *Fc* [a] per 10^7 cfu	*Qm*
48.0	2	5.64	63.3	219.0	114.3	5.6
27.0	1	5.30	56.1	129.0	122.7	5.9
25.0	3	1.59	130.5	269.0	74.5	4.0
24.0	2	3.09	79.9	98.0	41.5	2.7
9.0	1	1.89	85.7	31.3	20.6	1.8
3.0	1	1.39	113.0	19.7	5.0	1.2

[a] *Fc* Expt. 1, 55.3 mutants per plate, 25.1 mutants per 10^7 cfu.
Fc Expt. 2, 70.0 mutants per plate, 20.7 mutants per 10^7 cfu.
Fc Expt. 3, 94.7 mutants per plate, 38.3 mutants per 10^7 cfu.

TABLE 5

MUTATION TO A2C^r IN *E. coli* WITH TETRACYCLINE

Conc. of TC (μg/ml)	Expt. No.	Inhibition Total cfu con. / Total cfu Expt.	Percent survival cfu/plate Expt. × 100 / cfu/plate con.	*Fe* − *Fc* [a] per plate	*Fe* − *Fc* [a] per 10^7 cfu	*Qm*
9.0	1	30.69	14.5	−19.3	86.3	4.5
6.0	2	3.57	58.0	105.0	68.6	3.8
4.0	3	1.30	167.7	257.0	46.5	2.9
3.0	1	5.63	47.5	76.7	99.7	5.0
2.0	2	1.76	103.3	62.7	17.3	1.7
1.0	1	1.95	78.5	−7.0	2.8	1.1

[a] *Fc* Expt. 1, 55.3 mutants per plate, 25.1 mutants per 10^7 cfu.
Fc Expt. 2, 70.0 mutants per plate, 20.7 mutants per 10^7 cfu.
Fc Expt. 3, 94.7 mutants per plate, 38.3 mutants per 10^7 cfu.

TABLE 6

MUTATION TO A2C^r IN *E. coli* WITH GENTAMICIN

Conc. of GM (μg/ml)	Expt. No.	Inhibition Total cfu con. / Total cfu Expt.	Percent survival cfu/plate Expt. × 100 / cfu/plate con.	*Fe* − *Fc* [a] per plate	*Fe* − *Fc* [a] per 10^7 cfu	*Qm*
2.00	4	430.84	0.9	−15.2	1403.7	61.6
1.00	4	18.58	17.7	552.2	1165.8	48.0
0.80	3	2.15	77.9	71.3	47.9	2.9
0.60	2	2.38	66.0	11.0	15.6	1.6
0.50	4	1.86	87.2	60.8	26.8	2.1
0.45	1	5.52	46.2	133.7	158.7	7.4
	2	1.84	79.3	47.3	23.1	1.9
0.30	2	1.17	100.3	12.3	3.6	1.1
0.25	4	1.23	87.2	35.5	7.4	1.3
0.15	1	1.14	104.5	−8.3	−4.6	0.8
0.05	1	0.89	103.6	−13.3	−6.6	0.7

[a] *Fc* Expt. 1, 55.3 mutants per plate, 25.1 mutants per 10^7 cfu.
Fc Expt. 2, 70.0 mutants per plate, 20.7 mutants per 10^7 cfu.
Fc Expt. 3, 94.7 mutants per plate, 38.3 mutants per 10^7 cfu.
Fc Expt. 4, 56.0 mutants per plate, 19.4 mutants per 10^7 cfu.

TABLE 7

MUTATION TO A2C^r IN *E. coli* WITH OLEANDOMYCIN

Conc. of OM (μg/ml)	Expt. No.	Inhibition Total cfu con. / Total cfu Expt.	Percent survival cfu/plate Expt. × 100 / cfu/plate con.	*Fe* − *Fc* [a] per plate	*Fe* − *Fc* [a] per 10^7 cfu	*Qm*
2000.0	2	1.55	82.3	12.8	17.3	1.7
1000.0	3	2.58	71.6	3.7	28.1	2.1
500.0	3	1.99	109.6	36.7	13.5	1.5
300.0	1	1.25	90.3	56.7	25.5	2.0
100.0	4	1.19	84.6	−6.2	8.1	1.3
	1	1.40	96.3	−2.3	0.8	1.0
50.0	4	1.07	96.0	−44.5	−15.8	0.4
25.0	4	0.93	90.4	−47.2	−14.4	0.4

[a] *Fc* Expt. 1, 126.3 mutants per plate, 42.4 mutants per 10^7 cfu.
Fc Expt. 2, 109.9 mutants per plate, 41.7 mutants per 10^7 cfu.
Fc Expt. 3, 114.0 mutants per plate, 61.1 mutants per 10^7 cfu.
Fc Expt. 4, 150.5 mutants per plate, 60.0 mutants per 10^7 cfu.

TABLE 8

MUTATION TO A2C^r IN *E. coli* WITH PHOSPH NOMYCIN

Conc. of PM (μg/ml)	Expt. No.	Inhibition Total cfu con. / Total cfu Expt.	Percent survival cfu/plate Expt. X 100 / cfu/plate con.	*Fe* − *Fc* [a] per plate	*Fe* − *Fc* [a] per 10^7 cfu	*Qm*
20.00	2	45.52	21.6	−13.3	229.1	10.2
10.00	2	58.53	14.4	−4.0	409.3	17.5
5.00	3	5.28	84.5	370.4	301.9	13.2
2.64	1	1.04	131.2	−10.0	−5.2	0.8
0.66	1	0.72	130.8	22.0	0.5	1.0

[a] *Fc* Expt. 1, 22.3 mutants per plate, 9.0 mutants per 10^7 cfu.
Fc Expt. 2, 109.9 mutants per plate, 41.7 mutants per 10^7 cfu.
Fc Expt. 3, 114.0 mutants per plate, 61.1 mutants per 10^7 cfu.

TABLE 9

HISTIDINE REVERSION IN *S. typhimurium* TA98 WITH CHLORAMPHENICOL

Conc. of CAP (μg/ml)	Expt. No.	Inhibition Total cfu con. / Total cfu Expt.	Percent survival cfu/plate Expt. X 100 / cfu/plate con.	*Fe* − *Fc* [a] per plate	*Fe* − *Fc* [a] per 10^9 cfu	*Qm*
24.00	3	7.56	36.8	−4.0	2.5	1.2
12.00	3	6.08	39.3	8.3	19.3	2.5
9.00	2	4.06	58.1	16.3	14.8	2.1
3.00	2	1.58	91.8	4.3	2.1	1.2
1.50	1	1.31	114.1	8.0	1.3	1.1
0.50	1	1.15	133.7	0.3	−2.8	0.8
0.17	1	0.97	98.5	3.0	1.2	1.1

[a] *Fc* Expt. 1, 26.4 mutants per plate, 10.8 mutants per 10^9 cfu.
Fc Expt. 2, 29.4 mutants per plate, 9.0 mutants per 10^9 cfu.
Fc Expt. 3, 9.0 mutants per plate, 5.0 mutants per 10^9 cfu.

TABLE 10

PETITE INDUCTION IN *S. cerevisiae* WITH GENTAMICIN AT pH 4.4—4.7

Conc. of GM (μg/ml)	Expt. No.	Inhibition Total cfu con. / Total cfu Expt.	Percent survival cfu/plate Expt. X 100 / cfu/plate con.	*Fe* − *Fc* [a] per plate	*Fe* − *Fc* [a] per 10^3 cfu	*Qm*
4500.0	1	1.70	88.2	5.89	35.7	17.2
1500.0	1	1.65	94.4	4.44	25.1	12.4
500.0	1	1.41	117.8	2.67	11.9	6.4

[a] *Fc* Expt. 1, 0.22 mutants per plate, 1.18 mutants per 10^3 cfu.

TABLE 11

GENE CONVERSION IN *S. cerevisiae* WITH TETRACYCLINE AT pH 7.2

Conc. of TC (μg/ml)	Expt. No.	Inhibition Total cfu con. / Total cfu Expt.	Percent survival cfu/plate Expt. X 100 / cfu/plate con.	*Fe* − *Fc* [a] per plate	*Fe* − *Fc* [a] per 10^6 cfu.	*Qm*
4500.0	1	1.32	123.9	238.2	86.6	6.6
1500.0	1	1.71	109.2	204.8	90.9	6.9
1000.0	2	2.65	122.4	231.3	106.1	7.9
500.0	1	1.52	95.8	125.5	69.5	5.5
200.0	2	1.53	123.4	57.3	16.0	2.0
40.0	2	1.00	115.2	12.9	−2.1	0.9
8.0	2	0.83	148.7	38.6	−6.9	0.6

[a] *Fc* Expt. 1, 117.2 convertants per plate, 59.7 convertants per 10^6 cfu.
Fc Expt. 2, 113.4 convertants per plate, 71.6 convertants per 10^6 cfu.

TABLE 12

PETITE INDUCTION IN *S. cerevisiae* WITH TETRACYCLINE AT pH 7.2

Conc. of TC (μg/ml)	Expt. No.	Inhibition Total cfu con. / Total cfu Expt.	Percent survival cfu/plate Expt. X 100 / cfu/plate con.	*Fe* − *Fc* [a] per plate	*Fe* − *Fc* [a] per 10 [c]fu	*Qm*
4500.0	1	1.32	123.9	5.6	23.1	11.5
1500.0	1	1.71	109.2	0.4	2.1	2.0
1000.0	2	2.65	122.4	3.9	25.3	12.5
500.0	1	1.52	95.8	−1.0	−4.1	negative
200.0	2	1.53	123.4	0.1	−0.7	0.6
40.0	2	1.00	115.2	−0.1	−2.3	negative
8.0	2	0.83	148.7	0.1	−2.4	negative

[a] *Fc* Expt. 1, 2.17 mutants per plate, 5.1 mutants per 10^3 cfu.
Fc Expt. 2, 1.50 mutants per plate, 7.1 mutants per 10^3 cfu.

TABLE 13

MUTATION TO $A2C^r$ IN *E. coli* WITH ETHANOL

Conc. of EOH (μg/ml)	Expt. No.	Inhibition Total cfu con. / Total cfu Expt.	Percent survival cfu/plate Expt. X 100 / cfu/plate con.	*Fe* − *Fc* [a] per plate	*Fe* − *Fc* [a] per 10^7 cfu	Expt. muts. / Control muts. (per 10^7 cfu.)
188	4	2019.23	0.26	0.0	—	—
153	2	2416.67	0.20	0.0	—	—
133	2	406.16	1.19	−108.5	2.8	1.07
124	1	3.18	55.10	−15.3	−5.3	0.77
114	3	1.97	96.70	−34.0	−16.1	0.74
76	3	1.35	95.00	−49.3	−24.1	0.61
59	4	1.55	125.80	1.8	−11.7	0.81

[a] *Fc* Expt. 1, 26.7 mutants per plate, 18.1 mutants per 10^7 cfu.
Fc Expt. 2, 109.9 mutants per plate, 41.7 mutants per 10^7 cfu.
Fc Expt. 3, 114.0 mutants per plate, 61.1 mutants per 10^7 cfu.
Fc Expt. 4, 150.5 mutants per plate, 60.0 mutants per 10^7 cfu.

depends to some extent on the numbers of mutants counted [12]. However, unless the number of spontaneous mutants counted falls below 36 there is relatively little change in variance coefficient with change in mutation frequency. Thus the approximate significance can be estimated from variance coefficients (VC) calculated from duplicate negative controls [20].

$$VC = \sqrt{\frac{\Sigma d^2}{2n}}$$

d = differences between duplicate expressed as a % of their mean; n = number of duplicates.

Assuming a near-normal distribution of errors at each level of mutation frequency, mutation in experimental samples should exceed "E" when "n" = 4 (in the equation below) for certain significance ($p < 0.01$). Values for mutation less than E when $n = 2$ are unlikely to be significant ($p > 0.01$). Between these limits results are of doubtful significance and more tests may be needed to establish their status.

$$E = C \times \left[1 + \left(n \times \frac{\sqrt{1.5} \times VC}{100}\right)\right]$$

E = critical number of experimental mutants per 10^x colony-forming units (cfu); C = number of control mutants per 10^x cfu; $(\sqrt{1.5} \times VC)/100$ = average fractional standard deviation for the assay (with 2 control and one agent-treated samples).

Once significance was established from within-test controls, results were normalized to enable data to be compared directly [12]. However, instead of calculations relative to the average frequency (Q value), we have calculated results relative to the median frequency (Qm) of mutation.

$$Qm = \frac{Fe - Fc + Fm}{Fm}$$

Fe = experimental frequency in the assay; Fc = control frequency in the assay (a.v. of 2); Fm = median frequency (from accumulated data).

This change has been introduced for 2 reasons. The median will be less affected than the mean by the rare occurrence of very high spontaneous frequencies [8]. Furthermore, interval estimates on the median can then be given [4] for skewed distributions such as are found in liquid medium tests for spontaneous frequency.

Assessment of results of repair tests

Results from repair tests were expressed initially as inhibitions (I) relative to the negative control.

$$I = \frac{Nc}{Ne}$$

Nc = number of colonies from the negative control; Ne = number of colonies from the agent treated sample.

Inhibitions of strains WP100 and WP2 were then compared to give relative inhibitions (RI).

$$RI = \frac{I(\text{WP } 100)}{I(\text{WP } 2)}$$

The size of *RI* needed for significance can again be determined from variance coefficients calculated from duplicate negative control data as before.

Results

Optimization and use of microsomes

The optimal level of microsomes in the S9 mix for 2AF was 12.5 μl/plate (4.6 μl/ml) for BP, 25 μl/plate (9.3 μl/ml) and for CP, >200 μl/plate (74.1 μl/ml). Therefore, 2 levels of microsomes were used as a compromise in our tests, one high at 77.4 μl/ml and one low at 7.74 μl/ml. The responses with the 3 controls were consistent with those reported elsewhere [1,12].

With the antibiotics, microsomal activation was never essential to a positive response. Indeed most of the responses were unaffected or declined with microsomal addition.

Negative control frequencies, variance and significance

The accumulated data for negative control frequencies are shown in Table 1. It can be seen that the means are greater than the medians, usually by 1.5—2-fold. The interval estimates on the medians (at $p \triangleq 0.05$) are also quite wide as a result of the high variance. Nevertheless, the potential change in median is considerably less than that for the mean. (Furthermore the confidence limits for the medians for tests with *E. coli* will probably narrow as more data are accumulated.)

As expected [8,12,13] the variation in control frequencies between independent tests is large. However, variation within sets of tests is relatively small. From the variance coefficients, the average increases required for definite significance ($p < 0.001$) and for insignificance ($p > 0.05$) are shown in Table 1). Only the petite assay gives highly variable results; for the other tests the increases required for significance are small and quite similar for the different assays. The experimental negative control values (average of 2) are shown at the foot of the relevant tables.

Positive control values

All the positive control agents gave the expected degree of response. The types of genetic damage detected by reversion of *S. typhimurium* TA98 [10, 12], by mutation in *E. coli* [6,7,11,13] and by gene conversion in *S. cerevisiae* [5,11,13] have been defined in the literature.

Thus the positive controls used were designed to show that the assay was functioning normally. DR and ACR were used for *S. typhimurium* TA98 and MMS and ACR for *E. coli* WP2 and *S. cerevisiae* D4.

With petite induction and the repair test, it was considered necessary to define the range of detection and sensitivity of the assay systems. Therefore some further agents of known genetic activity were tested (Table 2), in addition to the controls, in assays with antibiotics. Both assay systems had a broad spectrum of detection of base-substitution and frame-shift agents. The repair test was particularly sensitive to highly bactericidal agents, e.g. MC, ACR and DR.

Toxicity and possible artefacts in tests

The antibiotics under test were all highly toxic to bacteria with the exception of the lack of lethality of OM to *E. coli*. Conversely there was little toxicity with yeast (even at 0.5 mg/ml) with any of the antibiotics.

The problems of selection and density-dependent growth of colonies have been discussed elsewhere for liquid culture assays [12,13]. However, we have evidence of an additional artefact of rare occurrence. At very low survivals there can be a density-dependent recovery of viability, i.e. at low plating densities survival can be apparently much lower than at high plating densities. As survivals are measured at 10^2 cells per plate and mutation at 10^9 cells per plate this can give an entirely spurious, large increase in mutation.

In view of these problems at low survival no result has been taken as positive in this paper unless there has been an actual increase in mutants per plate at some concentration of the agent even though there was no evidence of a survival artefact giving rise to spurious $A2C^r$ mutation with ethanol (Table 13). Unfortunately such data do not necessarily apply to antibiotics with a very different mode of action. However antibiotic treatment could not have caused a significant increase in spontaneous mutants by increasing growth of $A2C^s$ cells on selective agar because there was no visible increase in background growth relative to the controls.

Results of tests

Table 3 summarizes the results. All the antibiotics gave moderate to strong responses with forward mutation to $A2C^r$ in *E. coli* at or very near toxic concentrations with the exception of OM which was only weakly active and also relatively non-toxic (Table 4—8 and Fig. 1).

In addition, CAP gave a weak response with *S. typhimurium* TA98 (Table 9), GM was strongly petitogenic only at low pH (Table 10) and TC was significantly active for gene conversion and petite induction but only at high pH (Table 11 and 12 and Fig. 2). In the latter case a blackish precipitate was observed during the tests.

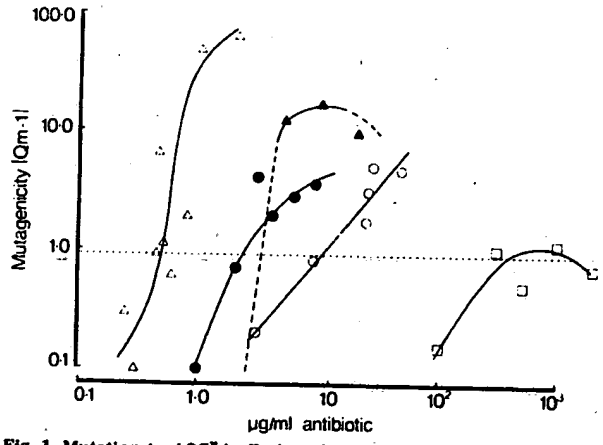

Fig. 1. Mutation to $A2C^r$ in *Escherichia coli* WP2. ○, CAP; ●, TC; △, GM; ▲, PM; □, OM.

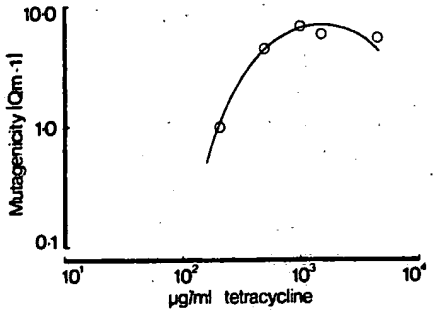

Fig. 2. Gene conversion with TC at pH 7.2.

The strongest responses were in forward mutation and many mutants were of small-colony phenotype. Furthermore, 4 of the antibiotics were protein inhibitors. It was therefore necessary to show that the mutants were of genetic origin rather than long-lived phenotypic variants.

On sub-culturing, about 10% of the smallest mutant colonies did not grow on selective agar. The rest were plated out on non-selective and selective agars and it was observed that survival was usually greater on non-selective media. This observation could be explained in 2 ways; either by segregation of sensitive cells or if the mutant colonies were of intermediate resistance (containing cells with a very short life-span on selective agar). To test which of these alternatives appertained, colonies from the non-selective agar were sub-cultured onto selective petri dishes. All the sub-cultures grew showing that no segregation was occurring, and hence that the mutants were genetically based and not phenotypic variants.

Mutation to $A2C^r$ only occurred at, or near, toxic doses. Thus there was a possibility that the production of mutants was a response of the system to *any* toxic effect and not peculiar to the agents under test. To eliminate this possibility tests were carried out with ethanol which showed no increase in mutants even at very toxic doses (Table 13). In fact a decrease of circa 25% was noted at most concentrations of ethanol which might indicate "anti-mutagenicity". It was also of interest that there was no correlation between decrease in viable cells and decrease in $A2C^r$ mutants per 10^7 colony-forming units.

Discussion

All the antibiotics tested (except oleandomycin) were potent mutagens for forward mutation to $A2C^r$ in *E. coli*. It was clear that this mutation was closely correlated with toxicity. Although it does not follow, of necessity, that the mutagenic and toxic effects were causally related, the inference must be that this was so in view of the diverse modes of action of the antibiotics tested.

Thus the evidence would seem to support the theory that antibiotics which affect bacterial metabolism are very likely to cause mutations albeit by an indirect mechanism. The fact that large effects were only seen in a forward-mutation assay implies that such mutations are almost exclusively deletions.

The lack of a positive effect in repair tests might imply that the deletion mu-

tations are mostly not repairable which seems unlikely. More probably, non-genetic toxic effects occur at lower doses than genotoxicity and completely mask any genetic response occurring in the repair test. An alternative possibility is that the antibiotics temporarily inhibit DNA repair.

It seems likely that petite induction by TC and GM could also be caused by deletion mutation in the mitochondrial DNA of *S. cerevisiae* (which is essentially similar to procaryotic DNA in organisation). The lack of effect of the other antibiotics might be related to penetration or possibly to weaker activity.

The response of the gene-conversion system in yeast to TC was unique in our tests. However, this response was achieved at pH 7.2, with aeration and in the presence of riboflavine (in the medium). All these factors favour TC degradation [9] which would account for the blackish precipitate seen in our tests.

These results clearly show that in the testing of compounds it is essential to use a variety of tester strains and genetic systems in order to obtain a true indication of *microbial* mutagenicity. In the case of antibacterial substances toxicity precludes the use of agar plate assays and liquid culture tests must be used in which survival can be measured [12].

However, the most significant conclusion to be drawn is that the testing of antibiotics in bacteria is a very dubious procedure. Not only is it hard to use realistic concentrations of agents but also the interpretation and evaluation of results is extremely difficult. Certainly in our tests, most (if not all) the positive results in bacteria can probably be attributed to the specific effect of the antibiotics on these organisms. Thus such results would be deemed to be inapplicable to mammals. Nevertheless both TC and CAP are suspected of involvement in carcinogenicity [18]; perhaps it is significant that these 2 compounds caused genetic effects not directly attributable to deletion mutation.

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